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Identification of Antifungal Substances of *Lactobacillus sakei* subsp. ALI033 and Antifungal Activity against *Penicillium brevicompactum* Strain FI02

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ABSTRACT: This study was performed to investigate the antifungal substances and the antifungal activity against fungi of lactic acid bacteria (LAB) isolated from kimchi. LAB from kimchi in Imsil showed antifungal activity against *Penicillium brevicompactum* strain FI02. LAB LI031 was identified as *Lactobacillus sakei* subsp. Antifungal substances contained in *L. sakei* subsp. ALI033 culture media were unstable at high pH levels. Both, the control and proteinase K and protease treated samples showed clear zones, suggesting that the antifungal substances produced by ALI033 were non-protein substances unaffected by protesases. Both, the control and catalase showed clear zones, suggesting that the antifungal metabolite was not H_2O_2. The molecular weights of the antifungal substances were ≤3,000 Da. The organic acid content of crude antifungal substances produced by *L. sakei* subsp. ALI033 showed high concentrations of lactic acid (502.47 mg/100 g). Therefore, these results suggest that antifungal substance produced by *L. sakei* subsp. ALI033 is most likely due to its ability in producing organic acid.

Keywords: kimchi, lactic acid bacteria, pathogenic fungi, antifungal activity, antifungal substances

INTRODUCTION

Fermentation of kimchi, a Korean traditional fermented food, is characterized by the generation of numerous lactic acid bacteria (LAB) as well as antibiotic active substances such as H_2O_2, CO_2, diacetyl, and bacteriocin (1). LAB produced during fermentation of kimchi inhibit growth of aerobic bacteria. *Leuconostoc mesenteroides* acidifies kimchi to maintain anaerobic conditions in the beginning stage, after which *Lactobacillus plantarum* strain is generated (2). A variety of metabolites generated by kimchi LAB have been used in functional beverages, foods, and diverse functional foodstuffs in order to improve nutrition or promote physiological activity. In addition, studies on the antibiotic and antifungal activities of LAB as natural food preservatives are being conducted (3).

Especially, strains isolated from kimchi have higher inhibitory activities, acid resistance, and bile tolerance than existing edible LAB preparations. Further, these strains inhibit proliferation of cancer cells and have excellent immune activity, inducing growth inhibition of harmful intestinal microorganisms and enhancing immune activity (4).

Preservatives (propionic acid, sodium propionate, and calcium propionate) as antifungal agents, ethanol as a chemical, and grapefruit seed extract, phytoncide, essential oils, and garlic as natural items were examined. As antibiotics, natamycin and Delvocid were investigated. Microbial agents, probiotics, *Propionibacterium*, bacteriocin, and kimchi LAB are known to be effective, and ozonization is known as an effective method to inhibit growth of mold (5-9).

In this study, LAB were isolated from kimchi in order to select bacteria with good antifungal activity and use them as basic materials for the development of antifungal preparations through evaluation of crude antifungal compounds and identification of molecular weight.

MATERIALS AND METHODS

Fungi indicator

Penicillium brevicompactum strain FI02 isolated from ripening cheese of the Imsil Research Institute of Cheese

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Science was used as the fungus indicator, incubated on potato dextrose agar (PDA) (Difco Laboratories Inc., Detroit, MI, USA) at 30°C for 2 d, and stored at 4°C. *P. brevicompactum* strain FI02 is the main fungus responsible for degrading the quality of Imsil cheese, so it was used to improve the quality of cheese by inhibiting fungi.

Isolation of LAB from kimchi

Kimchi samples were collected from homes in Jeonju, Imsil, Gwangyang, and Gyeonggido located in Korea. Screening for antifungal activities of LAB was performed as previously described (10). Kimchi samples were macerated using a hand blender (Hanil Electric, Seoul, Korea) for 2 min. The obtained kimchi juice was filtered through a sterile thin cloth, after which the filtrate was serially diluted with sterile-distilled water and then spread onto Lactobacilli MRS (de Man, Rogosa and Sharpe) agar (Difco Laboratories Inc.) + 2% $CaCO_3$. The plates were incubated at 37°C for 2 d, and tentatively considered LAB strains were selected. Among the selected strains, rod-type LAB were selected.

Antifungal activity assays

The paper disc assay (10) and the spot-on-the-lawn assay (11) were used to detect antifungal activities. Plates were prepared by adding mold (10^6 spores per 20 mL of PDA) up to a concentration of 1.5% (w/v). The spore solution was prepared as previously reported (12). For the paper disc assay, paper discs (diameter 8 mm; Advantec, Tokyo, Japan) on PDA plates were spotted with 100 μL of sample. The plates were incubated at 30°C for 48 h and examined for inhibition zones. For the spot-on-the-lawn assay, 10～25 μL of sample was spotted onto the sensitive mold plates. Antifungal activity was expressed as the clear zone size (mm). The above experiment was performed in triplicate.

Identification of the isolate

Analysis of the 16S rRNA gene sequence of LAB LI031 from kimchi in the Imsil region was performed by Macrogen, Inc. (Seoul, Korea) by the following method. BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosytems, Foster City, CA, USA) and 27F (5'-AGA GTT TGA TCA TGG CTC AG-3'), universal primer, as well as 1492R (5'-GGA TAC CTT GTT ACG ACT T-3') primer were used in the PCR reaction, and the sequence was analyzed using an ABI 3730×l DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The result was recorded by the 16S rRNA gene sequence comparative analysis and compared to the Gene Bank database in National Center for Biotechnology Information.

Isolation of antifungal substances

The crude antifungal substances were prepared as follows: *Lactobacillus sakei* subsp. ALI033 strain was incubated in Lactobacilli MRS broth (Difco Laboratories Inc.) at 37°C for 24 h. The culture medium was centrifuged (6,000 *g*, 15 min, Beckman Coulter, Fullerton, CA, USA) at 4°C to collect the supernatant, after which the crude antifungal substances were obtained by filtration through a 0.45 μm membrane filter (Merck Millipore, Billerica, MA, USA). The culture medium containing the crude antifungal substances was used in the experiment after concentrating it 10 times.

Effect of pH on antifungal activity

The effects of pH on the antifungal activities of the crude antifungal substances of *L. sakei* subsp. ALI033 were assessed. The pH of the crude antifungal substances of *L. sakei* subsp. ALI033 was adjusted to pH values of 3.0～8.0 using 1 N NaOH and 1 N HCl solutions and incubated at 37°C for 2 h, after which the growth inhibition against *P. brevicompactum* strain FI02 was measured using the paper disk method.

Activity assays upon proteinase K and protease treatment

The sensitivity of the antifungal substances to proteolytic enzymes was examined using the crude antifungal substances produced by *L. sakei* subsp. ALI033 in the medium. After incubation at 37°C for 2 h with 1 unit/mL proteinase K, the protease growth inhibition against *P. brevicompactum* strain FI02 was identified using the paper disk method.

Activity assays by catalase treatment

The crude antifungal substances produced by *L. sakei* subsp. ALI033 were based on H_2O_2, and 1 unit/mL of catalase (Sigma Aldrich Co., St. Louis, MO, USA) was added to the medium containing crude antifungal substances. The medium was reacted at 37°C for 2 h, and the growth inhibition against *P. brevicompactum* strain FI02 was identified using the paper disk method.

Antifungal activities of cell-free fractions

L. sakei subsp. ALI033 was incubated in 100 mL of Lactobacilli MRS broth (Difco Laboratories Inc.) at 37°C for 24 h, centrifuged (6,000 *g*, 15 min, Beckman Coulter) at 4°C to collect the supernatant, and filtered through a 0.45 μm membrane filter (Merck Millipore) to obtain the crude antifungal substances. The supernatant containing the crude antifungal substances was centrifuged (6,000 *g*, 15 min, Beckman Coulter) using a Centriprep YM-3 (Merck Millipore) and freeze-dried (Ilshin Bio Base, Seoul, Korea) by dividing into ≥3,000 Da and ≤3,000 Da fractions with molecular weights of 3,000 Da. The supernatant was then melted in 20 mM sodium acetate (pH 4.0) buffer (Sigma Aldrich Co.) and concentrated 10 times. The antifungal activities of the fractioned sam-

ples against *P. brevicompactum* strain FI02 were measured using the paper disk method.

Organic acids analyses

The crude antifungal substances produced by *L. sakei* subsp. ALI033 were withdrawn and centrifuged for 5 min at 10,000 rpm, and the supernatants were collected and filtered through membrane filters (Merck Millipore) with a pore size of 0.45 μm for the organic acid tests. Concentrations of the 6 main organic acids (lactic, citric, malic, succinic, acetic, and tartaric acids) were analyzed using a high performance liquid chromatography system (Waters M510, Waters Corporation, Milford, MA, USA) with a RSpak KC-811 column (ID 0.8×300 mm, Waters Corporation) operated at 25°C and UV 486 detector (Waters Corporation) at 220 nm. The mobile phase was composed of 95% (v/v) 3.3 mM KH_2PO_4 and 5% methanol with the pH adjusted to 2.5 with phosphoric acid, and the flow rate was set to 1.0 mL/min.

Statistical analysis

Data were expressed as mean±SD (standard deviation), and statistical analysis for single comparisons was performed using Duncan's multiple range test. Each experiment was repeated at least three times to yield comparable results. Values of $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

LAB isolation from kimchi

Forty four types of LAB were secured from kimchi collected from the different regions. Among the 44 types of LAB, 29 types of LAB separated from kimchi in Jeonju were named LJ001 to 029, 6 types of LAB separated from Imsil were named LI030 to LI035, 4 types of LAB from kimchi in Gyeonggi were named LGy036 to 039, and 4 types of LAB from kimchi in Gwangyang were named LGw040 to 044.

Antifungal activity of kimchi LAB

To determine the antifungal activities of the 44 types of LAB, the antifungal activity against *P. brevicompactum* strain FI02 was examined. These results suggest that LAB isolated from kimchi in Imsil and Jeonju exhibited antifungal activity against *P. brevicompactum* strain F102. Especially, LAB LI031 separated from kimchi in Imsil showed the highest inhibition zone. However, LAB isolated from kimchi in Gwangyang (LGw040～044) did not exhibit any antifungal activity (Fig. 1).

Identification of antifungal active LAB

LAB LI031 from kimchi in Imsil was identified as *L. sakei* subsp. ALI033 and used in this experiment. Magnusson et al. (13) reported that LAB such as *Lactobacillus coryniformis*, *L. plantarum*, *L. sakei*, and *Pediococcus pentosaceus* have antifungal activity, which is consistent with the results for *L. sakei* and *Pediococcus pentosaceus* in this study. Yang et al. (12) isolated antifungal-active LAB from kimchi in the same region but reported that *L. plantarum* AF1 exhibits antifungal activity against *Aspergillus*, which was not used in this study. On the contrary, in this study *Pencillium* isolated from cheese was used.

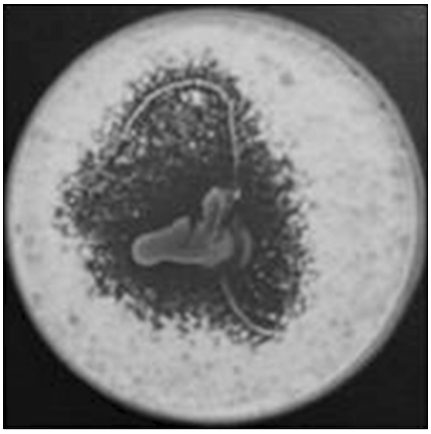

Fig. 1. Antifungal activity of lactic acid bacteria LI031 isolated from kimchi in Imsil against *Penicillium brevicompactum* strain FI02.

Antifungal activity at different pHs

To examine the effects of pH on *L. sakei* subsp. ALI033, antifungal-active LAB separated from kimchi were subjected to pH 3.0～8.0 using 1 N NaOH and 1 N HCl solutions, treated at 37°C for 2 h, and then examined for inhibitory activity against *P. brevicompactum* strain FI02 as described in Fig. 2a. The experiment was conducted based on the assumption that if the antifungal substance is an organic acid, the pH level will be low. Original activities were maintained at pH 3.0 and 4.0, whereas activities were not detected at pH 5.0～8.0. Considering these results, the antifungal substances contained in LAB culture media isolated in this study were unstable at high pH levels. Yang et al. (12) reported that substances produced by *L. plantarum* AF1 show activity at pH 3.0 and 4.0 but not at pH 5.0～7.0, which is consistent with the results of this study. Kim et al. (14) reported that antifungal substances produced by *Leuconostoc mesenteroides* CK0128, *Weissella cibaria* CK0633, and KK0797 produce non-protein antifungal substances with activities at pH 5 or below. Thus, the antifungal substances with activity in this study are also estimated to be non-protein substances.

Antifungal activity by proteinase K and protease treatment

To examine the sensitivity of the antifungal substances produced by *L. sakei* subsp. ALI033 to proteolytic en-

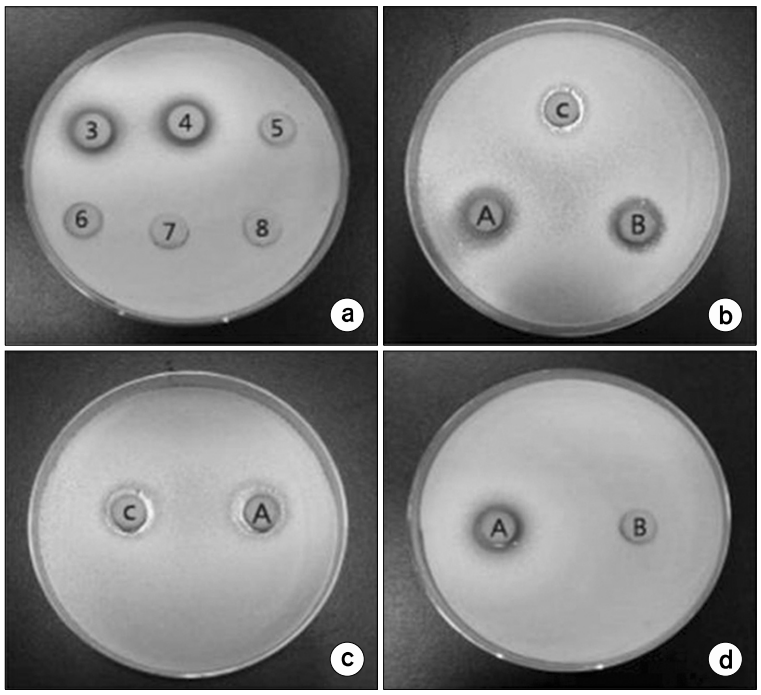

Fig. 2. Identification of antifungal substances of *Lactobacillus sakei* subsp. ALI033. (a) Effects of pH on antifungal activity of *L. sakei* subsp. ALI033. 3, pH 3.0; 4, pH 4.0; 5, pH 5.0; 6, pH 6.0; 7, pH 7.0; 8, pH 8.0. (b) Effects of enzyme treatment (proteinase K and protease) on antifungal activity of *L. sakei* subsp. ALI033. C, *L. sakei* subsp. ALI033 without treatment; A, proteinase K treatment of *L. sakei* subsp. ALI033; B, protease treatment of *L. sakei* subsp. ALI033. (c) Effects of catalase treatment on antifungal activity of *L. sakei* subsp. ALI033 culture medium. C, *L. sakei* subsp. ALI033 without treatment; A, catalase treatment of *L. sakei* subsp. ALI033. (d) Antifungal activities of two cell-free fractions from *L. sakei* subsp. ALI033 separated by 3,000 Da molecular weight. A, fraction with molecular mass lower than 3,000 Da; B, fraction with molecular mass higher than 3,000 Da.

zymes, proteinase K and protease were treated at a concentration of 1 unit/mL to measure growth inhibition against *P. brevicompactum* strain FI02. The results are described in Fig. 2b. Both, the control and proteinase K and protease samples showed clear zones, suggesting that the antifungal substances produced by *L. sakei* subsp. ALI033 were non-protein substances unaffected by protesases. Lee et al. (15) reported that the antifungal metabolites of LAB isolated from neonatal feces and dongchimi (water-based radish kimchi) are not proteinaceous substances. Chung et al. (16) reported that the antifungal substances produced by the KK3 strain are proteins or peptidergic bacteriocin, which are different from the results of this study. These results are likely different since the subject of microbial inhibition was fungus in this study but food poisoning bacteria were used in the study by Chung et al. (16).

Antifungal activity by catalase treatment

The antifungal substances produced by *L. sakei* subsp. ALI033 strain were based on H_2O_2, and catalase was used at a concentration of 1 unit/mL to measure the growth inhibition against *P. brevicompactum* strain FI02. The results are described in Fig. 2c. The metabolites driving the antifungal effects of LAB were reported to be organic acid, H_2O_2, and bacteriocin (17). As H_2O_2 is degraded upon catalase treatment, the inhibition ability was identified after catalase treatment. Both, control and catalase showed clear zones, suggesting that the antifungal metabolite was not H_2O_2. Lee et al. (15) reported that antibacterial ability was not eliminated, which is similar to the results of the current study.

Comparison of the antifungal activities by molecular weight of antifungal substances

The antifungal effects of the fractioned samples were measured as in Fig. 2d. The activity on *P. brevicompactum* strain FI02 was not observed at ≥3,000 Da, but was detected at ≤3,000 Da, suggesting that the molecular weight of the antifungal substances was ≤3,000 Da. The properties of metabolites with antibacterial and antifungal effects isolated from kimchi in the studies by Yang et al. (12) and Kim et al. (14) are similar to the results of the current study.

Organic acids

The organic acids content of the crude antifungal substances produced by *L. sakei* subsp. ALI033 showed high concentrations of lactic (502.47 mg/100 g) and acetic (158.75 mg/100 g) acids. Other organic acids, including

Table 1. Composition of organic acids in the crude antifungal substances produced by *Lactobacillus sakei* subsp. ALI033 (mg/100 g)

Organic acids	Crude antifungal substances
Acetic acid	158.75±18.8[b1)2)]
Citric acid	35.46±3.2[c]
Lactic acid	502.47±31.3[a]
Malic acid	2.46±0.2[d]
Succinic acid	–
Tartaric acid	59.54±4.1[c]
Total	758.68

[1)]Mean±SD.
[2)]Values with different letters (a-d) within a column are significantly different ($P<0.05$) by Duncan's multiple range test.

citric, malic, and tartaric acids were also detected in the same supernatant (Table 1). Park et al. (18) reported that the organic acids content of *L. paracasei* strains showed high concentrations of lactic (113 mg/100 g) acids. Therefore, lactic acid production of *L. sakei* subsp. ALI 033 strains was 4.5 times higher than that of *L. paracasei* strains.

CONCLUSION

Our results suggest that the antifungal substance produced by *L. sakei* subsp. ALI033 is most likely due to its ability to produce organic acids. Also, *L. sakei* subsp. ALI 033 may help to improve the quality of cheese by inhibiting *P. brevicompactum* strain FI02.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

REFERENCES

1. Son HK, Lee MY, Chang HC, Lee JJ. 2013. Acute toxicity of crude anti-fungal compounds produced by *Lactobacillus plantarum* AF1. *J Korean Soc Food Sci Nutr* 42: 892-897.
2. Lee H, Lee JJ, Chang HC, Lee MY. 2012. Acute toxicity of *Lactobacillus plantarum* AF1 isolated from kimchi in mice. *Korean J Food Preserv* 19: 315-321.
3. Ko KH, Liu W, Lee HH, Yin J, Kim IC. 2013. Biological and functional characteristics of lactic acid bacteria in different kimchi. *J Korean Soc Food Sci Nutr* 42: 89-95.
4. Park YH, Ryu BH, Lee IS, Kang KH. 2006. Acid tolerant probiotic *Lactobacillus sakei* Probio-44 that can suppress the growth of pathogenic microorganisms and suppressed tumour growth and promoted the production of several different cytokines of immune responses. *Korea Patent* 10-0574527 -0000.
5. Sitara U, Niaz I, Naseem J, Sultana N. 2008. Antifungal effect of essential oils on *in vitro* growth of pathogenic fungi. *Pak J Bot* 40: 409-414.
6. Sridhar SR, Rajagopal RV, Rajavel R, Masilamani S, Narasimhan S. 2003. Antifungal activity of some essential oils. *J Agric Food Chem* 51: 7596-7599.
7. Suomalainen TH, Mäyrä-Mäkinen AM. 1999. Propionic acid bacteria as protective cultures in fermented milks and breads. *Le Lait* 79: 165-174.
8. Tagg JR, Dajani AS, Wannamaker LW. 1976. Bacteriocins of gram-positive bacteria. *Bacteriol Rev* 40: 722-756.
9. Pintado CMBS, Ferreira MASS, Sousa I. 2010. Control of pathogenic and spoilage microorganisms from cheese surface by whey protein films containing malic acid, nisin and natamycin. *Food Control* 21: 240-246.
10. Yang EJ, Chang HC. 2008. Antifungal activity of *Lactobacillus plantarum* isolated from kimchi. *Kor J Microbiol Biotechnol* 36: 276-284.
11. Hoover DG, Harlander SK. 1993. Screening methods for detecting bacteriocin activity. In *Bacteriocins of Lactic Acid Bacteria*. Hoover DG, Steenson LR, eds. Academic Press, Inc., San Diego, CA, USA. p 23-39.
12. Yang EJ, Chang HC. 2010. Purification of a new antifungal compound produced by *Lactobacillus plantarum* AF1 isolated from kimchi. *Int J Food Microbiol* 139: 56-63.
13. Magnusson J, Ström K, Roos S, Sjögren J, Schnürer J. 2003. Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. *FEMS Microbiol Lett* 219: 129-135.
14. Kim HR, Lee JH. 2013. Selection of acid-tolerant and heterofermentative lactic acid bacteria producing non-proteinaceous anti-bacterial substances for kimchi fermentation. *Korean J Microbiol Biotechnol* 41: 119-127.
15. Lee JY, Park YS, Kim YS, Shin DH. 2002. Antimicrobial characteristics of metabolites of lactic acid bacteria isolated from feces of newborn baby and from *Dongchimi*. *Korean J Food Sci Technol* 34: 472-479.
16. Chung JH, Bae YS, Kim YJ, Lee JH. 2010. Characteristics of bacteriocin produced by a *Lactobacillus plantarum* strain isolated from *kimchi*. *Kor J Microbiol Biotechnol* 38: 481-485.
17. Oliveira PM, Brosnan B, Furey A, Coffey A, Zannini E, Arendt EK. 2015. Lactic acid bacteria bioprotection applied to the malting process. Part I: Strain characterization and identification of antifungal compounds. *Food Control* 51: 433-443.
18. Park KT, Oh MH, Nam JO, Ji KB, Han JK. 2014. Characterization of mutant strain, *Lactobacillus paracasei* ML-7 isolated from kimchi, and its effect on the growth of broiler. *Korean J Food Sci Technol* 46: 148-152.